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Review

Role of small bioorganic molecules in stem cell differentiation to insulin-producing cells

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Abstract—The use of small specific molecules has been instrumental in the modulation of stem cell proliferation and differentiation to obtain insulin-containing cells. Examples include nutrients (glucose, nicotinamide and retinoic acid), acids (butyrate), alkaloids (cyclopamine and conophylline) and pharmacological agents (LY294002 and wortmannin). These molecules, alone or in combination with specific growth factors and hormones, will likely provide key information to design specific culture media in order to obtain customized cells for implantation in diabetes. In addition, the study of such molecules will help to understand the mechanisms involved in stem cell biology as well as contribute to the design of specific drugs for islet repair and regeneration in diabetes.

Contents

1. Introduction

Diabetes is characterized by the presence of abnormally high and sustained levels of glucose circulating in the blood stream. Under normal circumstances, insulin is released from the endocrine pancreas in response to a rise in glycaemia, such as after a meal. The hormone stimulates glucose removal from the blood stream to muscle and fat tissue, allowing the recovery of normoglycaemia.[1] Diabetic patients produce little or no insulin, favouring the presence of high levels of circulating su-

Abbreviations: ASCs, adult stem cells; DMEM, Dulbecco's modified Eagle's medium; EBs, embryoid bodies; ERK, extracellular signal-regulated kinase; ESCs, embryonic stem cells; K_{ATP} channel, ATP-dependent potassium channel; LIF, leukaemia inhibitory factor; MODY, Maturity onset diabetes of the young; NAD^+, nicotinamide adenine dinucleotide; PARP, poly(ADP-ribose) polymerase; Pdx-1, Pancreatic duodenal homeobox-1 transcription factor; PI3K, phosphoinositide 3-kinase; RA, all-*trans*-retinoic acid; Shh, Sonic hedgehog.

Keywords: Stem cells; Diabetes; β-Cell; Insulin secretion.

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gar. Insulin is produced by a specific cell type (the β-cell) located within the endocrine pancreas, inside cell aggregates called islets of langerhans. These structures contain other specialized cell types such as α-cells, which produce glucagon, δ-cells, which produce somatostatin, and PP-cells, which produce pancreatic polypeptide.

According to the underlying causes, diabetes can be classified mainly in two groups: type 1 and type 2. Type 1 diabetes is an autoimmune disease in which β-cells are the targets of the immune system. The onset of this disorder is attributed to both genetic and environmental factors, such as diet or an early infection.[1] Antibodies against proteins of the β-cell, such as anti-glutamate decarboxylase and anti-tyrosin phosphatase, have been described. The disorder affects mainly children and young people and represents 5% of the total cases of diabetes. Type 2 diabetes, corresponding to the 95% of the diabetic population, is a more complex pathology often associated with obesity and overweight.[2] The disease occurs mainly in adults, presenting a progression from insulin resistance to failing production of the hormone by β-cells. MODY (maturity onset diabetes of the young) is a monogenic subtype of diabetes mellitus characterized by young onset, abnormalities of β-cell function and autosomal dominant inheritance. Some forms are closer to type 2, but others (i.e., MODY-1 and MODY-3) are more severe.[1]

Although absolute insulin dependence is required for survival in type 1 diabetic patients, hormone administration is not totally necessary in the case of type 2 diabetes.[1] However, daily insulin injections are a palliative method that cannot mimic the exquisite work that β-cell itself exerts in the control of body glycaemia. This lack of regulation favours the development of long-term complications, such as neuropathy, nephropathy, retinopathy and cardiovascular disorders.[1] Replacement or regeneration of the damaged β-cells or islets are strategies to consider in the treatment of diabetes. The classical double transplantation of pancreas and kidney represents a major surgery approach. More recently, a transplantation protocol of islets of Langerhans obtained from cadaveric donors has been established.[3] Aside complications related to immune-suppressor administration, this intervention has set up the basis for a specific replacement protocol in the treatment of the disease. However, the main problem to face in these surgical procedures is the scarcity of cadaveric pancreata, limiting the use of this protocol to a reduced number of patients. Therefore, new cell sources are necessary for future replacement strategies, and embryonic as well as adult stem cells offer very promising possibilities.[4]

2. Stem cells

Stem cells possess two fundamental properties: the ability to self-renew indefinitely in culture, and, under the appropriate extracellular stimuli, specialize into specific cell fates.[5] Stem cells are classified in two main groups depending on their origin: embryonic and adult. Embryonic stem cells (ESCs) are derived from the inner cell mass of the blastocyst and under appropriate signals give rise to all cell lineages of the individual, including the germ line.[5,6] Adult stem cells (ASCs) are tissue-specific and usually committed to mature cells of the tissue in which they are located.[7] Both ESCs as well as ASCs can proliferate over long periods of time, although this property is more restricted in ASCs. Mouse ESCs can be maintained undifferentiated in vitro by adding leukaemia inhibitory factor (LIF) to the culture medium.[8] In any case, multiple rounds of division in culture can lead to chromosomal abnormalities and altered pattern of gene expression in both cell types.[9,10] The differentiation plasticity of ASCs is restricted to specific cell fates compared to ESCs, although several laboratories argue that ASCs can transdifferentiate to a greater variety of cell types when placed in the appropriate niches.[11] This finding could offer new possibilities for developing alotransplantation protocols, but the ability of ASCs to fuse with mature cells in the new niche, has questioned the true potential of these cells.[12] In any case, it seems that cell fusion is a very unusual event. Mouse ESCs can spontaneously differentiate when grown in suspension in the absence of LIF, forming typical cell aggregates called embryoid bodies (EBs).[8] These structures give rise to cell lineages of the three primary embryonic germ layers: ectoderm, mesoderm and endoderm.

Stem cell differentiation towards specialized fates is controlled by intracellular and extracellular determinants that have the ability of modulating specific gene programmes. These determinants include biophysical interactions such as cell-to-cell contacts and cell-matrix signals, peptide and protein-related molecules such as growth factors, hormones and cytokines, and low molecular weight molecules which include nutrients, metabolites, acids, natural products and synthetic pharmacological agents.[13] The effect of these determinants, the optimal concentration, the appropriate mixture of factors and the time of addition to the extracellular media still represent main challenges in tissue bioengineering.[14] These instructive environments could be instrumental in the design of protocols for in vitro directed differentiation, decreasing the number of spontaneous differentiation events which occur in monolayer or in EBs, and leading to homogeneous cell populations.

Insulin-positive cells have been described during spontaneous differentiation in EBs cultured for more than 10 days.[15] However a detailed protocol to obtain pure populations of insulin-secreting cells from ESCs is still lacking. The main problems to face in this sense are the uptake of insulin from the culture medium and the presence of neuroectodermal as well as primitive endoderm-derived populations, which are also positive for the hormone.[16,17] Cells of hepatic origin, such as oval and foetal liver cells, can also be manipulated to induce insulin expression.[18,19] However, these cells tend to co-express other pancreatic hormones such as glucagon, suggesting that further maturation protocols should be developed in order to use liver biopsies in replacement protocols. On the other hand, the pancreas harbours quiescent stem cells though it is still in debate

where they are located. In this sense, exocrine tissue, duct epithelium and the same β-cells are potential sources of new insulin-producing cells.[20–22] Therefore, the identification of the islet precursor population and the design of protocols for in vivo and in vitro expansion and differentiation are key challenges in this area.[23] Another alternative source for insulin-producing cells is ASCs from mesoderm-derived tissues such as bone marrow and circulating monocytes.[24,25] Pancreas repopulation from highly purified bone marrow stem cells has been described, although the mechanisms implicated in this process have not been fully elucidated.[24,26] Reprogramming blood monocytes in the presence of cytokines (interleukin-3 and macrophage colony-stimulating factor), and subsequent exposure to growth factors (epidermal and hepatic growth factors) as well as nicotinamide, allows the obtention of insulin-containing cells. However, the yield of this protocol varies between individuals.[25]

Bioorganic molecules have provided efficient chemical tools to control cellular processes by modulation of metabolism, signal transduction pathways and gene regulation. Some of these compounds have been instrumental in other differentiation protocols, such as all-*trans*-retinoic acid in obtaining neuronal cells or 5-azacytidine in skeletal muscle differentiation.[27,28] In this sense, certain molecules (Fig. 1) have been used by various investigators in order to drive the differentiation of stem cells towards a β-cell-like phenotype, as well as to promote the proliferation and differentiation of β-cell precursors isolated from adult tissues. The present review will focus in those exogenous compounds used in different protocols as well as in the possible mechanisms involved in the differentiation towards insulin-positive cells. Potential and future perspectives of these compounds in this field will be also discussed.

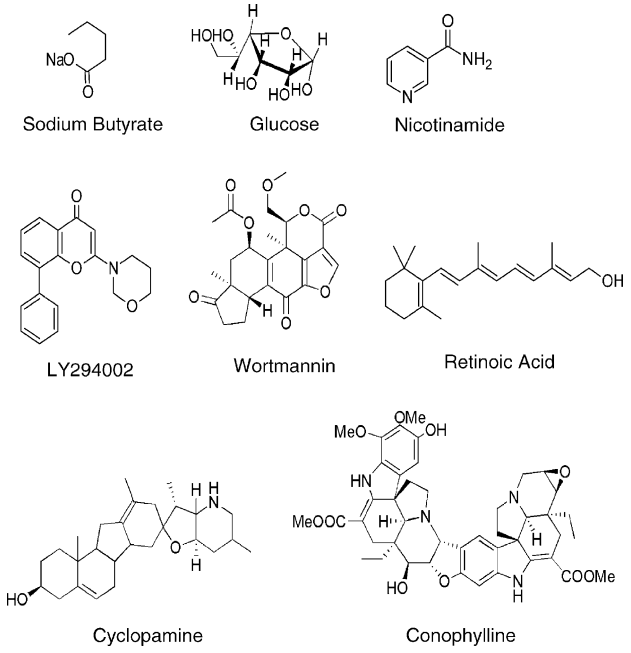

Figure 1. Chemical structures of the small molecules discussed in this review.

3. Nutrients

3.1. (a) Glucose

Acute glucose elevation is the key event in the induction of insulin secretion. To this end, glucose has to enter in the β-cell through the glucose transporter GLUT1 (humans) or GLUT2 (rodents), and be quickly metabolized by glucokinase in the glycolytic pathway.[29] This augmented glycolytic activity leads to an increase in the metabolic flux through the Krebs cycle, elevating thereby the ATP/ADP ratio and diadenosin-polyphosphate concentration.[30,31] This rise produces the closure of ATP-dependent potassium (K_{ATP}) channels, subsequent plasma membrane depolarization and the opening of voltage-dependent L-type calcium channels.[32] The increase in intracellular calcium would activate proteins involved in the exocytotic pathway inducing insulin release.[33] Aside from this well-known physiological effect on insulin secretion, the sugar can produce β-cell dysfunction and impaired insulin secretion under conditions of sustained hyperglycaemia, typical of diabetic states. This is due to the fact that, besides its calorigenic function, glucose is capable of modulating different gene programmes, producing key phenotypic changes in the β-cell that lead to cell dysfunction, dedifferentiation and culminate in cell death.[34–39] Therefore, glucose is a key metabolite not only in regulating insulin secretion, but also in maintaining a differentiated stable phenotype in pancreatic β-cells.

The prominent role played by glucose in β-cell pathophysiology prompted us to consider this nutrient as an instrumental component in the differentiation protocols developed in vitro to obtain insulin-producing cells. The working glucose concentration in Dulbecco's modified Eagle's medium (DMEM) used in stem cell experiments is 25 mM, which is five times higher than the physiological concentrations found in normoglycaemic conditions.

At high concentrations and in undifferentiated cells, glucose could act as a mitogenic agent, providing the required energy for cell divisions. In this sense, we have incubated D3-ESCs at different glucose concentrations ranging from 5 to 25 mM and we have observed that the proliferation rate was dependent on sugar concentration. However, the differentiation state, according to the pattern of marker gene expression, was not altered under the different culture conditions. Similar observation was noticed in neuronal stem cells in which the lowering of glucose during the proliferation period diminished epidermal growth factor-dependent proliferation.[40]

On the other hand, and taking into account the information provided from studies in β-cells, we suspect that glucose could influence the plasticity of stem cells in order to acquire a mature phenotype. In our first protocol,[41] we used a cell selection strategy to obtain insulin-producing cells based on the resistance to an antibiotic under the control of the regulatory regions of the insulin gene. The lowering of glucose concentra-

tion from 25 mM (typical of DMEM) to 5 mM in the last step of this protocol has allowed to increase the insulin content of cells derived from mouse ESCs.[41] The operating molecular mechanisms have not been addressed, but it is believed that normoglycaemia might normalize expression levels of glucose-regulated genes, which are linked to correct glucose sensing, hormone secretion and maintenance of β-cell phenotype. Furthermore, another report describes the in vitro differentiation of insulin-positive cells from human ESCs based on the selection of nestin-precursors developed in mice.[42] The incubation in normoglycaemic conditions at the end of the protocol has been instrumental to improve the yield of insulin-containing cells. However, the authors of this publication claimed that the final product was not fully mature islet cells, because they display low responsiveness to glucose in terms of secretion and the cells presented co-expression of insulin along with other pancreatic endocrine hormones, such as glucagon and somatostatin.[43] In agreement with these observations, the incubation of neuronal stem cells in the presence of low glucose during the differentiation period favoured the neurogenesis and astrogliogenesis.[40]

The presented evidence supports a prominent role for glucose in bioengineering protocols which deserves more attention, in particular when obtaining nutrient-sensitive cells such as neurons, hepatocytes and β-cells.

3.2. (b) Nicotinamide

Nicotinamide (also known as niacinamide) is a form of vitamin B3, which through its major metabolite NAD^+ (nicotinamide adenine dinucleotide) is involved in a wide range of biological processes, including the production of energy, nutrient metabolism, signal transduction, as well as maintenance of the integrity of the genome.[44–46]

The antidiabetogenic potential of nicotinamide is well known, as it has been seen in several animal models of type 1 diabetes, such as streptozotocin-treated as well as pancreatectomized mice and rats.[47–49] Though the mechanisms of this anti-diabetogenic effect are not well known, several hypotheses have been established, such as the scavenging of free radicals or the reduction of the immune damage on β-cells.[50,51] In this sense, in vitro studies have shown that nicotinamide can inhibit the expression of MHC class II antigen in islets,[52] preventing macrophage infiltration or interleukin-1β-induced damage.[53] In addition, nicotinamide has been shown to decrease the population of interleukin-12 and tumour necrosis factor-α production in cultures of whole blood from prediabetic and diabetic subjects, as well as in healthy controls.[54]

The results with nicotinamide obtained from clinical trials in humans, however, have not been successful, suggesting that those effects are not the main operating pathways for this compound. In 1993, it was reported that nicotinamide enhanced the in vitro differentiation of cultured human foetal pancreatic cells, favouring the expression of insulin, glucagon and somatostatin.[55] The mechanism was mainly attributed to the inhibition of the poly(ADP-ribose) polymerase (PARP), since inhibitors of this enzyme were capable of mimicking the effect of nicotinamide as well. This could also explain the results observed in animal models in which β-cell destruction is mediated by chemical agents and not by autoimmune attack.

PARPs are a large family of 18 enzymes that catalyze a broad post-translational modification in which more than 200 units of ADP-ribose are added successively onto target proteins.[56] Those proteins are mainly histones and other nuclear proteins involved in DNA repair and synthesis, transcription and cell cycle control.[57,58] This modification is transient but extensive, since PARPs use large amounts of NAD^+, compromising the energy status in the cell. In this sense, eukaryotic cells have developed proteolytic mechanisms to protect from activated PARPs. In apoptotic processes, the sudden induction of widespread DNA damage leads to an excessive activation of the enzyme, decreasing intracellular ATP levels necessary for the culmination of the suicide programmes. In this context, PARP cleavage by specific caspases is an early event during apoptosis. Consequently, nicotinamide could increase the intracellular NAD^+ pool, thus allowing cell survival.[57,59]

More recently, the differentiation potential of nicotinamide has been exploited in bioengineering protocols to obtain insulin-producing cells. In all cases tested so far, nicotinamide has been an instrumental factor to produce in vitro insulin-positive cells either from ESCs and ASCs.[21,41,60–65] The molecular mechanisms in which nicotinamide exerts its function in these protocols remain to be explored, but it can be hypothesized that this compound could be related directly or indirectly to survival and DNA repair of injured proliferating cells and chromatin remodelling, allowing the activation of specific gene programmes. Altogether, nicotinamide seems to be an indispensable culture component in the last stages of in vitro differentiation protocols towards insulin-producing cells.

3.3. (c) Retinoic acid

Due to its key role for ectoderm and mesoderm development in vivo,[66] all-*trans*-retinoic acid (RA) has been extensively used in protocols designed to promote neuronal differentiation in vitro.[27,67] Recently, new evidence supported a role of RA signalling in early pancreas differentiation from embryonic endoderm.[68] Based on this idea, RA was used in bioengineering protocols to obtain insulin-producing cells. The addition of RA on the 4th day after EB formation promoted the commitment to endoderm precursor cells.[69] Under these conditions, cells express early endoderm markers, but not insulin or glucagon genes, suggesting that RA can facilitate in vitro commitment to pancreatic precursors. However, the addition of RA the 1st day of EB formation did not enhance insulin expression.[70] Both apparently contradictory results correlate to the commitment to endoderm that occurs late in EBs (around days 5–7). On the other hand, the combination of RA with activin A in a simple

three-step approach allowed the development of differentiated cells expressing insulin.[71] Although the mechanisms operating in these differentiation processes have not been studied in detail, it is believed that the targeting of RA to nuclear receptors to activate specific gene programmes may be the most probable effects.[72]

4. Acids: butyrate

Butyrate is an organic acid that has been previously reported to increase the differentiation potential of foetal pancreatic cells,[73] as well as induce insulin secretion in islets and β-cell gene expression.[74–76] However, cells treated with sodium butyrate have increased apoptosis.[77] This effect, while beneficial for the treatment of insulinomas,[78] is detrimental for developing proper in vitro differentiation protocols. The combination with nicotinamide, which promotes cell survival, seems to overcome this problem.[72] The effect of this compound is still unknown, but it could act as an inhibitor of histone deacetylase, controlling thereby gene expression, cell proliferation and differentiation.[79]

5. Alkaloids

5.1. (a) Cyclopamine

In vitro studies have shown that the alkaloid cyclopamine, isolated from plants of the Veratrum genre, promotes pancreatic differentiation in the developing stomach and duodenum.[80] Cyclopamine possesses a steroid structure related to cholesterol, and it is known to block the membrane protein Smoothened, which mediates the cellular response to Sonic hedgehog (Shh).[81] The inhibition of the binding of Shh to its receptor Patched is a key event for differentiation and morphogenesis of the pancreas.[82] On the other hand, endoderm-derived organs, such as liver, stomach and duodenum, require this signalling pathway. During embryonic development, pancreatic Shh repression is performed by factors released from the notochord, which are activin β-B and fibroblast growth factor-2.[82] The blockage of this pathway allows expression of the transcription factor Pdx-1, which plays distinct roles. At early stages (embryonic day 9.5–10), Pdx-1 is required for pancreas development, whereas in later stages (embryonic day 18) this transcription factor is instrumental in β-cell differentiation, and in the adult pancreas is a key factor in GLUT-2 and insulin gene expression. Mutations in this transcription factor are related to the development of an inherited form of diabetes called MODY 4.[83]

Conversely, when Shh binds to Patched, Smoothened is activated and produces a cascade of signals resulting in the inhibition of pancreatic differentiation.[84] Therefore, in vitro direct blockade of Shh signalling pathway can allow islet differentiation. This can be achieved by using antibodies against Shh that impair Shh-Patched interaction, or by cyclopamine administration, which acts directly by blocking Smoothened. This results in induction of Pdx-1 and insulin expression, as it has been demonstrated in differentiation protocols of mouse ESCs.[64,70]

5.2. (b) Conophylline

Conophylline is a vinca alkaloid extracted from the tropical plant *Ervatamia microphylla*. This compound acts as well in the Shh pathway, mimicking the effect of activins repressing Shh expression.[85] In addition, activin A, when bound to its receptor, can activate p38 mitogen-activated protein kinase inducing the expression of neurogenin3.[82]

Conophylline addition to pancreatic rudiments in culture as well as to AR42J cells (a model of pancreatic progenitors) increased the number of insulin, and Pdx-1-positive cells. Treatment with conophylline of neonatal streptozotocin diabetic rats increased as well the number of insulin-positive clusters budding from ductal structures. This results in glucose normalization and improved glucose tolerance for 2 months.[85] The data support the role of conophylline in β-cell regeneration, which has also been described in the same animal model treated with glucagon-like peptide and exendin 4.[86]

6. Pharmacological agents: LY294002 and wortmannin

Phosphoinositide 3-kinases (PI3Ks) are involved in numerous cellular processes, including apoptosis, proliferation, cell motility and adhesion. Their primary function is to phosphorylate phosphoinositides at the 3-hydroxyl moiety, and the resulting molecule acts as a membrane docking for proteins such as protein kinase B and phospholipid-dependent kinase 1.[87] This enzyme is specifically inhibited by LY294002 (2-(4-morpholin, 1)-8-phenyl-4*H*-1-benzopyran-4-one hydrochloride) and wortmannin, representing thereby powerful tools to study the implication of this transduction pathway in several cellular processes.

In the pancreatic β-cell, it has been suggested that PI3K signalling is modulating several cell functions, including the regulation of the K_{ATP} channel, the voltage-dependent calcium channel as well as exocytosis, all associated to glucose metabolism.[88] Its involvement has also been shown in the autocrine insulin feedback action, where insulin through the insulin-like growth factor-I receptor would initiate signal transduction cascades, which are most likely linked to cell proliferation processes. In this context, it has been previously shown that PI3K inhibition promotes differentiation of pancreatic foetal cells, increasing insulin mRNA, content and secretion.[89] In this sense, both LY294002 and wortmannin have also been used in conjunction with nicotinamide to mature mouse embryonic stem cell-derived insulin-producing cells.[61]

Recently, it has been shown that mouse embryonic stem cell self-renewal can be regulated by LIF-activated PI3K-dependent signalling.[90] The implication of this pathway has been proven by using LY294002, resulting

in a hampered self-renewal capacity.[90] Although the signalling networks are not fully clarified, it seems that PI3Ks are implicated in a negative regulation of extracellular signal-regulated kinase (ERK) activity in ESCs. PI3K inhibition favours ERK activation and thereby differentiation versus proliferation events. This effect differs from observations in differentiated cells, where inhibition of PI3Ks results in reduced ERK activity.[91]

7. Future perspectives

The potential use of stem cells for the treatment of diabetes needs to overcome significant obstacles. Many studies have focused on the role of small peptides, cytokines and proteins in stem cell differentiation to obtain insulin-producing cells.[14] In addition, several investigators have used genetic modifications in conjunction with differentiation factors to obtain insulin-producing cells. Blyszczuk et al.[92] used a vector that over-expressed the transcription factor Pax4 on embryonic stem cells, followed by the differentiation protocol developed by Lumelsky.[60] As a result they obtained cells positive for insulin, as well as expression of other pancreatic factors such as Pdx-1, Ngn3 and GLUT-2. The cells responded to increase in glucose concentration in vitro and ameliorated hyperglycaemia for 14 days when transplanted into diabetic mice.

However, little is known about the role of small bioorganic molecules in such processes (Fig. 2). Bioengineering protocols require better characterization of the signal transduction pathways that control specific gene programmes leading to particular cell fates. Small compounds may offer the ability to manipulate those pathways, allowing a precise regulation of in vitro differentiation and proliferation events.

Screening for novel compounds could be complemented with cell-based assays, including genomic and proteomic characterization,[93] such as the use of microarray or DNA chip technology. As of yet, no specific β-cell DNA chip has been developed, however there are stem cell-specific chips. Perez-Iratxeta et al.[94] collected Affymetrix DNA microarray data of over 200 samples, including stem cells and their derivatives. These data are being used to genetically characterize the stem cells, being available online (StemBase; http://www.scgp.ca:8080/StemBase/).

Therefore, chemical structures can be bioengineered in order to design molecules that are specific of particular cell events related to proliferation, differentiation and function. To this end we need to take in account several issues:

(a) Specificity of action versus pleiotropic effects, that is, compounds that reproduce growth factor events without side effects.
(b) How close is the cell phenotype obtained by the small molecule compared to the desired phenotype.
(c) Toxic effects in terms of time and dose when used in the different protocols.
(d) Potential in combination with other compounds and determinants.

In addition to in vitro bioengineering of ESCs, there are other fields in which new treatments of diabetes could find interesting options. These include in vitro reprogramming of ASCs and in vivo regeneration, as well

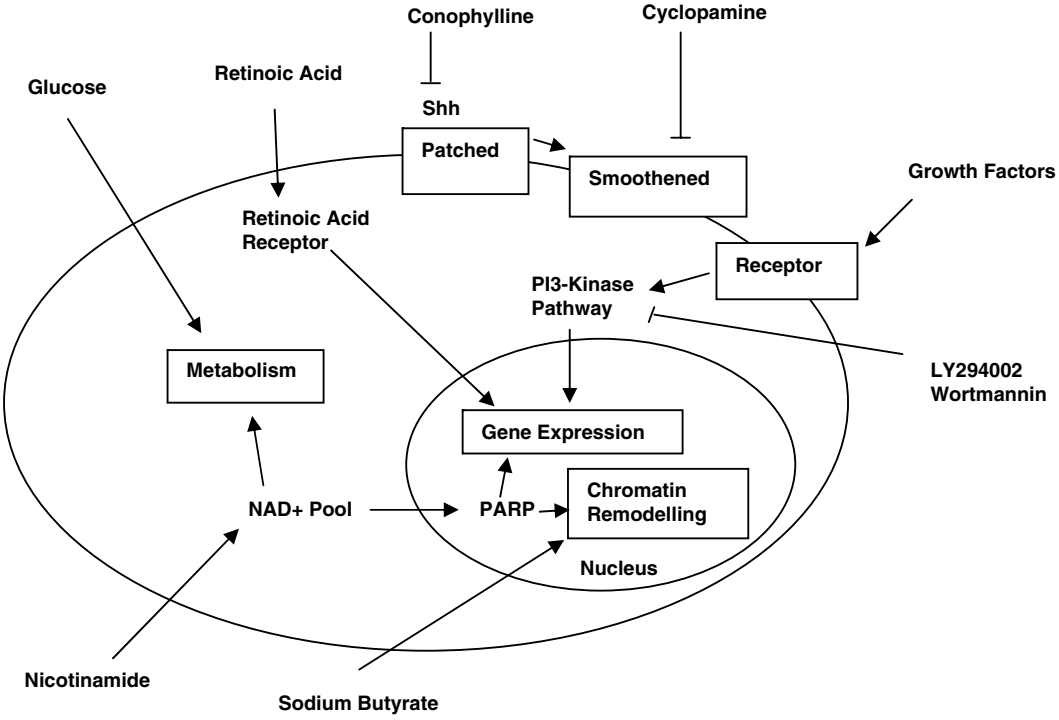

Figure 2. Possible mechanisms of action of certain small molecules in obtaining insulin-producing cells from stem cells.

as improving survival of the endocrine pancreas. Although we are still in an early stage of protocol development, this is important information demonstrating that small compounds have the potential to modulate stem cell differentiation to insulin-secreting cells, opening new perspectives in diabetes treatment.

In conclusion, it must be noted that despite the efforts and major advances that have been made in the field, the use of small molecules solely in the process of stem cell differentiation will not give likely the desired result. Combination with other strategies must be considered, such as genetic modifications (gene over-expression or silencing), proper culture conditions utilizing proper serum concentrations and lot specificity, as well as appropriate differentiation factors at a determined concentration and exposure time, cell selection processes, etc. All must be taken in account to develop viable strategies. Thus, the combined use of certain factors, including well-designed small molecules, in specific protocols would allow deriving efficiently stem cells to insulin-secreting cells.

Acknowledgments

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